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THE RATE OF YEAST MASS SYNTHESIS IN CONTINUOUS FODDER YEAST PROPAGATION

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The theory of continuous bacterial cultures proceeds from the assumption of their homogeneity. The rate constant of propagation expresses the rate of protein mass accumulation based on a unit of protein mass content of the culture in conditions of a given experiment. This rate constant does not represent a permanent value, but depends on the flow rate. The mechanism of rate constant self-regulation at flow rate alterations has not been sufficiently elucidated.

During fodder yeast propagation the culture is not homogeneous. There are always cells present which are budding, resting or dying off. All of them take a more or less active part in protein mass accumulation. The kinetics of protein mass accumulation by the *Candida utilis* yeast may be conceived as an autocatalytic reaction with product yield as an exponential function of the original substance amount entering the reaction. In the biosynthetic reaction not all of the protein mass takes part, but only the active, viable cells.

We suggest a method for experimental determination of protein mass synthesis rate constant and of the active yeast amount in *Candida utilis* propagation in a batch culture. The independence of this constant from changes in substrate concentration and of the amount of seed yeast has been shown. But the constant depends on the nature of the substrate and on the presence of biostimulators. Changes in substrate concentration and yeast amount, all other conditions being unchanged, lead to variations in the active yeast content of the culture. Therefore the self-regulating mechanism of yeast growth rate in a continuous culture at flow rate changes is conditioned by alterations in active yeast content, but the protein mass synthesis rate constant remains unchanged. In a continuous yeast cultivation on mixed media, containing a mixture of different sugars, the degree and rate of their assimilation are governed by the same kinetic laws as batch processes.

In a complex medium with a flow rate ensuring, at a given concentration of yeast, the glucose content assimilation, xylose and other not easily assimilated sugars will not be utilized by the yeast. To achieve better utilization of the remaining sugars (xylose, galactose, arabinose, etc.), either the flow rate is to be diminished or the yeast concentration raised. It is to be considered, however,

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that an increase of the yeast concentration decreases their activity. Therefore it is often more profitable to dilute the substrate and to increase the flow rate.

The presence in the medium of dissolved oxygen and its rate of dissolution plays an important rôle in fodder yeast propagation as a limiting factor. An increase in the oxygen saturation rate of the medium augments the yeast protein mass yield, as related to the sugar consumed, and the amount of active yeast.

At yeast propagation in nonsterile conditions highly productive yeast are often displaced by less productive ones, the propagation of yeast and the degree of carbon-containing substrate assimilation being diminished. The phenomenon may be also due to a lesser activity displayed by the productive yeast in a combined cultivation with other yeasts or yeastlike microorganisms.

INDUSTRIAL WASTE WATER TREATMENT BY MEANS OF CONTINUOUS FERMENTATION BY SUL-  
PHATE-REDUCING BACTERIA

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Bacteria of the genus *Desulfovibrio* have been used in treating some kinds of industrial waste water in the ČSSR. The object of this research work was to alter the existing spontaneously fermenting processes of biological treatment to conveniently controlled processes with all the requirements of an industrial fermentation procedure. Naturally, this could be performed only on the basis of a thorough knowledge of the physiology and biochemistry of the organisms employed and of the given substrate, namely the waste water, and its influence on the microorganism. Such a controlled fermentation process for biological treatment was largely successful in the case of waste waters from baker's yeast and citric acid production. The new technique using the sulphate-reducing bacteria was termed "sulphate-reducing fermentation method". The principles of this method for the waste waters mentioned above may be summarized in the following points:

- (1) Selected and adapted cultures of *Desulphovibrio rubentschickii* are grown on substrates consisting of waste water with the addition of phosphorus nutrients and cca 0.2% sodium sulphate. With the seed thus prepared a system of gas-tight chambers was inoculated in the ratio 1 : 15 to 1 : 20.
- (2) The fermentation is performed in two stages. The sulphate-reducing bacteria in a symbiotic culture, for example with bacteria of the type *Clostridium*, *Aerobacter* etc., were introduced into the first stage, where complex organic matter /betain, protein, reducing matter etc./ is degraded into simpler substances /organic acids/. In the second stage, where the culture *Desulfovibrio* already predominates, an intensive sulphate reduction proceeds accompanied by the degradation of the remaining organic matter, and hydrogen sulphide and carbon dioxide develops. At this stage the fermentation proceeds in the presence of metallic iron as iron shavings, acting as biocatalyst and favouring the redox potential of the medium.
- (3) The inflow of the waste water into the gas-tight fermentation system must be continuous. The fermentation is conducted at 28 - 35°C, the pH values 6.5 - 6.8, under continuous stirring of the bacterial mass, preferably

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by gas. During the fermentation the gas products are constantly removed so that a moderate underpressure arises throughout the system.

- (4) Some deficient material, e.g., 0.1 - 0.3% waste sodium sulphate or in the case of waste water from citric acid fermentation a small amount of superphosphate and ferrous sulphate may be added into the waste water.

The fermentation time was substantially decreased in comparison with the still existing biological methods, e.g., the methane fermentation and in the case of yeast production waste water it is only 48 hours (compared with 6 days) and with waste water from citric acid production 4 - 5 days (compared with 14 to 16 days). The purifying efficiency is substantially higher and in either case the purification degree reaches 80 - 85%, expressed in BOD, 80% according to the oxydative permanganate test lasting 4 hours, 90 - 95% betain nitrogen, 90 - 95% sulphates, 80 - 84% total sulphur etc. Gases containing as much as 20% hydrogen sulphide, 45 - 50% methane and 15% carbon dioxide develop during the fermentation.

The present sulphate-reducing fermentation method which has been not only tested on plant scale but also applied in a number of cases in practice, seems to be promising even for other kinds of waste water. Besides, it offers the advantage of utilizing valuable products from the biological treatment processes.

PREDICTION OF FLOW RATE FOR CONTINUOUS FERMENTATIONS OF PHENOSOLVAN-EXTRACTED  
WASTE WATERS FROM BROWN COAL PYROLYSIS BY ACTIVATED SLUDGE

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For our experiments the same waste water was used as described by Leibnitz and Schulze in the report "Studies for Continuous Fermentation of Phenosolvan-extracted Waste Waters from Brown Coal Pyrolysis by a Strain of *Rhodotorula glutinis*".

It was fortified with  $H_3PO_4$  in the same manner.

An activated sludge was used, received from the biological waste water purification plant of Lauchhammer. For maintenance of activity the sludge was cultivated in an aeration vessel continuously fed with diluted waste water.

In batch experiments the best results were obtained by using a waste water diluted to a fatty acid concentration of 2 gr/litre. Higher concentration experiments showed complete inhibition of growth.

Under optimum conditions the maximum growth rate constant was determined to  $0.16 \text{ h}^{-1}$ .

Continuous experiments were accomplished in the same apparatus as described by Leibnitz and Schulze. The rate of aeration excluded any oxygen limitation. The same start-technique was used, too.

It was found that the fatty acid concentration of added waste water can be increased up to 5.5 gr/litre without notable increase of fatty acid concentration at steady state. Furthermore, the system was stable within dilution rates up to  $0.22 \text{ h}^{-1}$ .

These results prove the remarkable fact that under conditions of continuous flow the attainable growth rate is much higher than in batch experiments although substrate levels measured as fatty acid concentrations are reversed.

These results are due to toxic effects of some substrate components.

Therefore, the prediction of maximum growth rate and other parameters being a function thereof does not lead to exact values using maximum growth rate constants obtained from batch fermentations.

In every case of substrates containing toxic components the maximum growth rate has to be determined by continuous experiment itself.

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A NEW VERTICAL-TYPE APPARATUS FOR CONTINUOUS ALCOHOLIC FERMENTATION

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The conversion of a batch fermentation system to a continuous technique requires the solution of a number of microbiological problems as well as a new technical and engineering equipment.

With these aspects we approached our work. Firstly, attempts were made to reduce the contamination necessarily occurring in the course of molasses fermentation. The contamination was induced by heterofermentative strains of lactic acid bacteria, namely, *Lactobacillus buchnerii*, *L. vermiformis*, *L. leichmanii* and others. As inhibiting agents antibiotics and disinfectants were used, Aureomycine showed to be the most effective and entirely eliminated the contamination within 14 days of fermentation. Formaline additions of 0.02 g/100 ccm reduced the infection to 50%, relative to control. In the course of fermentation this amount was gradually increased above the limiting concentration without interfering with the fermentation. Increments of 0.01 g per 100 ccm added in 48 hours' intervals kept the contamination in the initial state for 14 days. If the widely recommended sodium pentachlorate was used special care had to be taken that it should not interfere with the fermentation process especially, when the yeast strains were reused according to Boinotte.

Further studies were devoted to the design of a new turbulating fermentation equipment vertically arranged and tested on 1/10 of plant scale. This equipment may be used not only for continuous alcoholic fermentation but after a slight modification even for the production of organic acids, solvents, etc.

This apparatus facilitates full utilization of the operating space. The system is tightly closed and eliminates the unfavourable effect of carbon dioxide on the agitation of the fermenting liquor and consequently on the uniform distribution of the specific weight.

The apparatus consists of vertically arranged fermentation vessels divided by plates into a number of sections. The plates are fitted with caps allowing the fermenting liquor to flow only in one direction (from the bottom to the top); the carbon dioxide evolved during the fermentation acts in the caps as a kind of gas valves.

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Continuous yeast propagation and continuous pitching of the system with returned fermented liquor was tested in this apparatus additionally fitted with automatization.

In the first case, when 20<sup>0</sup>Bg molasses was fed into the fermentor and the whole content of the apparatus was changed in 24 hours, the average yield obtained amounted to 58 - 59% based on sugar added.

In the second case, samples of the fermenting liquor were taken from the fermentation system at a point where the yeast showed their optimum properties (under approximately the same experimental conditions) and about 61.5% yields based on sugar added were obtained. This method proved to be simple in operation although greater care was to be taken to prevent the fermentation from contamination.

It will be the subject of future investigations why the yields based on sugar added were lower in continuous systems compared with those obtained in batch fermentations.



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# A COMPARATIVE STUDY OF CELL MASS PRODUCTION IN A SINGLE- AND MULTI-STAGE CULTIVATION

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The present communication records a comparative study of the yeast mass production in a single- and two-stage continuous cultivation with regard to the maximum utilization of the substrate and of the working volume of the fermentor. The relation of the volumes of the two fermenting vessels ( $v$ ) is given by the expression

$$V_2 = \frac{\mu_1 x_1}{x_2 (D_2 - \mu_2)} V_1,$$

where  $\mu$  is the specific growth rate,  $x$  is the concentration of microorganisms and  $D$  is the dilution rate. The indexes 1 and 2 refer to the first and second fermentor, respectively. For the production in a two-stage system  $P'_2$  and two parallel single-stage systems  $P'$  it holds that

$$P'_2 = \mu_1 x_1 V_1 + \mu_2 x_2 V_2 \quad \text{and}$$

$$P' = \mu_2 x_2 V_2 + \mu_2 x_2 V_2,$$

providing that the final concentration of the microorganisms in the outflow is in either case  $x_2$  and the concentration of the substrates in both outflows is identical.

The computed relations were experimentally tested with the yeast genus *Torulopsis* and sucrose used as the only carbon source limiting growth.

The single-stage system proved to be under the conditions of fermentation preferable to the two-system for its better utilization of the substrate and of the working volume of the fermentors. In the two-stage cultivation either lower yields or lower utilization of the working volume were obtained. These lower yields are brought about by two different kinds of sugar metabolism. At the concentration of 0.5 mol inverted sugar in the medium of the first fermentor the formation of esters besides the cell mass was observed.

Further studies of the changes, occurring in the computed relations if a mixture of two sugars utilized in diauxy is used as limiting factor, were made and the repressive effects of glucose on the formation of an adaptive enzyme system investigated.

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ON THE PRODUCTION OF ERGOT ALKALOIDS IN A CONTINUOUS PROCESS

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The replacement culture technique has been employed the first time by Abe et al. Brady and Tyler to produce ergot alkaloids in saprophytic culture.

In order to improve this method we modified for our studies an apparatus, which has been originally developed by Kuška for the continuous cultivation of Mycobacteria.

This procedure is especially suitable for the study of the physiology of the alkaloid formation.

We were able to show that it is possible to produce ergot alkaloids with one mycelial mat over a long time in a semi-continuous process.

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THE POLAROGRAPHIC DETERMINATION OF OXYGEN UPTAKE AND TRANSFER RATE IN AEROBIC  
STEADY-STATE YEAST CULTIVATION ON LABORATORY AND PLANT SCALE

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The measurement of the actual oxygen uptake rate by microorganisms at a given instant of the fermentation process by means of the polarographic dropping mercury electrode is a rather complicated matter. The sulphite oxydation method determining the oxygen solution rate is relative, cannot be used in the presence of microorganisms or nutrient media and is not feasible on plant scale. The actual values of these variables under ordinary conditions may be conveniently, exactly and reliably measured by means of the polarographic technique with solid electrodes plated with polyethylene or teflon foils.

A system with Pt-Ag/AgO electrodes of long term stability and a total compensation of the temperature coefficient with a simple stable direct current supply and a transistor amplifier of the measured current has been developed and allows registration and control with ordinary apparatuses. The solved oxygen concentration (C) and the actual oxygen uptake rate (Q) by the microorganisms under the conditions of fermentation in a fermenting vessel of arbitrary capacity may be measured with this device.

The instant values  $C_2$  and Q may be measured by means of *Saccharomyces cerevisiae* or *Candida utilis* cultures at a favourable dry weight concentration under steady-state conditions according to the following equation:

$$Qx = K_L a (C - C_L) .$$

If x (yeast dry weight concentration) and C (oxygen solubility under the conditions given) are known, the constant  $K_L a$  characterizing the efficiency of the aeration equipment and the oxygen transfer rate under the actual conditions of fermentation may be calculated. The values of the oxygen transfer rate thus obtained are of great importance for the design of aeration systems for aerobic fermentations. The method may be applied to various aerobic microorganisms, various physical and physico-chemical conditions of nutrient media and may serve to test the efficiency of aeration equipment in fermentation vessels of arbitrary capacity.

The accuracy of the method depends on the precise determination of Q and there-

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fore particular attention has been paid to the determination of this variable in the fermenting vessel itself or by means of sampling.

The aeration equipment of laboratory fermentor of 1.5 l capacity was tested and the results compared with those obtained with the sulphite method determinations. The actual oxygen transfer rate in the continuous cultivation of *S. cerevisiae* in the molasses medium was 30% lower than that given by the sulphite method. Another measurement was made during the continuous cultivation of *S. cerevisiae* in molasses medium in a 300 hl fermentor fitted with different aeration systems. The dissolved oxygen concentration was measured in the fermenting vessel by means of a submerged electrode and registered. From the known oxygen solubility data  $C$  and the measured values  $C_L$  and  $Q$ , the values  $K_L a$  and  $K_L a C$  were calculated for the aeration system of perforated tubes, that of Phrix, Vogelbusch and the system of the turbine impeller at various rotations per hour and varying amounts of air. Simultaneously, the power used for stirring was measured and the power used for the amount of the air supplied was calculated. From these values the calculations of the air uptake for the output of 1 kg of yeast solids, of the power used for stirring and aerating and of the percentual utilization of the air supplied were made. The results showed a considerable economic advantage of the turbine impeller system requiring the power of 0.4 - 0.9 kW for 1 kg output of yeast solids with the present experimental arrangement over the system with perforated tubes requiring the power of 1.4 kW/kg and utilizing 4 - 9% of the air supplied under the same conditions. Of the stirred systems the one with the turbine impeller deserves special attention requiring the power of 0.4 - 0.6 kW and  $5.5 \text{ m}^3$  of air for 1 kg of yeast solids formed at a 45% air utilization in the optimum case, whereas the Phrix and the Vogelbusch systems showed the requirement of 0.55 - 0.93 kW/kg and 0.49 - 0.59 kW/kg and the maximum air utilization 24% and 11%, respectively.

Finally, the method was tested in the continuous *C. utilis* cultivation in a 1000 hl fermenting vessel fitted with the aeration system with perforated tubes. The results are in fairly good agreement with the actually obtained maximum constant dry weights which show the beginning of the oxygen limitation.

Substituting this relation in equation /5/ and integrating we obtain

$$t = \frac{1}{k} - \frac{1}{\rho} \ln \frac{c}{c_0} \quad /8/$$

$$\text{As } D = \frac{1}{t} \quad /9/$$

Equation /8/ enables us using equation /9/ to calculate with sufficient precision the size or performance of the first chemostat, presuming that no sediment of microorganisms is present (or that there is no dead space due to imperfect construction).

Equations /5/ to /9/ were successfully used for the calculation of quite complicated apparatuses for continuous alcoholic fermentation (with the recycling of medium, with considerable sediment etc.).



USE OF CONTINUOUS CULTURE METHOD FOR ANALYSING THE CELL FUNCTIONS

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A live cell resembles a perfect self-adjusting machine. It responds to external influences within its capacities by a reconstruction of its structures and functions, aimed at preserving viability under changes conditions. Irreversible processes of differentiation arising in the cell, it evades the student's control. But in other cases the physiological state of the cell remains stable, except the cyclically repeating changes related to the cell-division. The cell-medium complex might then be considered as a balanced system amenable to precise control. A certain morpho-physiological state of the cell corresponds to every complex of external conditions. This can be achieved by cultivating cells in flowing, continuously refreshed media (according to the homogeneous continuous method). When the heterogeneous-continuous (gradient) method is used, both the medium composition and the physiological state of the cells change as the liquid moves through the apparatus. Such a method is continuous only in the technological, but not in the physiological aspect.

The growth conditions in a cultivator depend both on the composition of the medium employed and on the density of the cell population. As the flow of the medium is slowed down, the density of population rises, the concentration of the limiting factors increases and the growth rate of cells is retarded until it becomes equal to the dilution rate. The limiting factors might be represented by nutrients concentration, metabolic products or pH. The relationship between many such factors and growth rate of cell is given by Michaelis' equation. This permits to predict the rate of growth and substrate consumption when the process parameters change. The relationship between the limiting factors and the population density is not so constant.

Two types of functional-analytical investigations are possible. In some cases the composition of the initial medium is made to vary, without changing its flow rate (and, consequently, the growth rate). In this way it is possible to ascertain the effect of individual external factors on the morphology of the cell, its chemical composition (notably, the DNA and RNA content) and biochemical activity (respiration intensity, biosynthesis of adaptive enzymes and pharmacologically active substances, etc.). By repeating investigations at various flow rates, it is possible to differentiate the phenomena related or

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CONTRIBUTION TO THE THEORY OF CONTINUOUS FERMENTATION

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All biological processes take place in time, but independently of time. The rate of fermentation depends mainly on the amount of microorganisms (N), or on the amount of microorganisms in the unit of volume (n), concentration of nutrients (c), concentration of metabolites (m), temperature (T), pressure (p), etc. We can, therefore, say that the time (t) necessary for the fermentation process is the function of the above mentioned factors.

$$t = f(n, c_1, c_2, c_3, \dots, m_1, m_2, \dots, T, p, \dots)$$

Considering that the metabolite is formed from the nutrient we can write:

$$m = \varphi(c)$$

Under constant pressure and temperature and using one type of nutrient as the limiting factor, the equation is reduced to:

$$t = f(n, c) \quad /3/$$

by differentiation we obtain:

$$dt = \frac{\partial t}{\partial n} dn + \frac{\partial t}{\partial c} dc \quad /4/$$

Experimentally it was proved that

$$\frac{\partial t}{\partial n} = -\frac{1}{k n} \quad \text{and} \quad \frac{\partial t}{\partial c} = \frac{1}{c^c}$$

We can then write relation 4 as follows:

$$dt = -\frac{1}{k n} dn + \frac{1}{c^c} dc \quad /5/$$

By integrating we obtain

$$t = -\frac{1}{k} \ln \frac{n}{n_0} + \frac{1}{c} \ln \frac{c}{c_0} \quad /6/$$

This equation is valid not only for batch fermentation, but also in some cases for continuous fermentation; equation /5/, however, is generally valid.

Relation

$$dn = Ddt \quad /7/$$

is also valid for the first chemostat, where D is the dilution rate.

not related to the growth rate.

In the second case the concentration of but one factor is made to vary. Simultaneously the flow rate is changed in order to keep the cell number at one and the same level, all the external factors remaining constant except the varied one. This makes possible to find the quantitative dependence between the concentration of certain substances (nutrients, vitamins, antiseptics, etc.) and the cell growth rate.

The report gives some data illustrating the above propositions.

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YEAST FLOCCULATION AND SORPTION IN CONTINUOUS SULFITE WASTE LIQUORSFERMENTATION

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It was observed that prolonged fermentation of sulfite waste liquors by the continuous method results in the selection and accumulation of grainy and sorbing yeast in the system. Most important in natural selection is the role of cell flocculation and their sorption by the solid phase media. As these properties of yeast are of a general theoretical interest, they merit to be discussed in more detail. It is assumed that yeast cells possess an aqueous membrane, an electric charge and exist as a whole unit. We may assume that in the presence of such depressing dehydrating substances as Ca, Mg and Fe ions and ethyl alcohol, the cell integrity is affected: they lose the aqueous membrane and the electric charge is lowered which hinders their coupling at a mutual collision. When the cells get near one another their polysaccharide membranes with many hydrophalous groups become more compact due to the fixation and compact packing of water molecules in their micelles. Thus single cells flocculate forming lumps or grains. In the presence of sorbents, e.g. cellulose fibres, the cells with diminished electric charges are affected by the same forces of coupling as in flocculation. Therefore the presence of aqueous membranes of cells and their water content play a decisive role in flocculation and sorption phenomena.

The conclusions are based on determinations of the water content in powder flocculating and sorbing yeast grown on different media. The total water content of yeast cells was determined by yeast drying at 105°C. Free or osmotically mobile water was estimated by yeast plasmolysis in a saturated solution of NaCl. Water fixed in the cells has been determined by subtraction of the free water content from the total water content. By approximation we may say that in powder yeast the relation of free water content to the dry matter and to the fixed water is 2 : 1 and 1 : 1 respectively. The contrary is observed in flocculating and sorbing yeast. It was found that flocculation and sorption are accompanied by a decrease of free water in the cells and by the increase of fixed water. The growth stimulators lead to the production of cells with an increased free water content and depressing substances affect a decrease of free water in the cells and an increase of fixed water. On the basis of nitro-

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gen content of powder and flocculating yeast we may assume that water fixation depends on a high polysaccharide content of the cells.

For industrial use of flocculation and sorbing yeast a new fermenter design based on yeasts selfcondensation was developed.

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SIMPLE STOCHASTIC MODELS OF A MULTISTAGE CONTINUOUS CULTURE OF MICROORGANISMS

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The purpose of the present paper is to give an approximating stochastic model of the growth (in time) of a culture of microorganisms that are continually cultivated in several mutually joint vessels through which the culture medium is flowing.

Although it would be principally possible to take into consideration even more complicated, in time varying conditions, in the present paper the author deals only with the case when the conditions in every vessel are independent of time (i.e. in the steady state).

First, the process in question is supposed not to be of Markov type and the influence of this assumption on first and second order characteristics is briefly treated. The influence having been shown inessential, in the remainder of the paper the flow cultivation is studied on a Markovian model as a multiparameter birth, migration and death process with several parameters.

Though the starting point are the differential - difference equations for the probabilities of the culture being in fixed states, the calculations are confined to the determination of first and second order moments.

In particular the time-increase of the relative dispersion indicating the increasing instability of the culture is studied and in simple cases also the extinction probability is given.

## LACTIC ACID PRODUCTION BY CONTINUOUS FERMENTATION AND ISOLATION METHOD

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The trend for higher effectivity of the fermentation industries requires the application of continuous processes. Naturally, the continuity must be consistently followed throughout the production cycle, i.e., from the preparation of the mash to the isolation of the finished product.

Various branches of the fermentation industry, e.g., yeast factories, distilleries etc. have adopted not only the continuous technique of fermentation but even the isolation of the biomass produced or of the corresponding metabolites is conducted continuously.

In the case of lactic acid, however, in spite of several attempts for a continuous lactic acid fermentation, the isolation of this acid from the fermented liquor has always been a periodical process.

The object of our present research work was therefore not only to solve the problems of a continuous lactic acid fermentation production, but at the same time to study the continuous lactic acid isolation directly coherent with the control of the fermentation process.

In model experiments, a medium made up of refined sugar with supplementary nutrients was used. The level of the nutrients added was investigated not only from the viewpoint of the requirements of the producing organisms but also with regard to the difficulties in the isolation of the acid and finally to the economic profit.

The fermentation was conducted in a three-vessel fermentor the mash being fed into the first member of the arrangement which had the double capacity of the two remaining vessels. During the fermentation the acid produced was neutralised by means of calcium carbonate additions.

The results obtained established that

- (1) the culture used showed its greatest activity in the first stage of fermentation utilizing as much as 75% of the sugar present;
- (2) the fermentation time (consequently the flow-rate) depended on the amount of supplementary nutrients and ranged from 2 to 6 days at the initial

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sugar concentration 5%;

- (3) alike the periodical fermentation the continuous method gave higher yields and fuller conversions to lactic acid with lower initial sugar concentrations and yields might reach 93% based on sugar added;
- (4) in some cases under favourable experimental conditions a larger amount of by-products was formed indicating a certain degree of degeneration of the microorganisms whose appearance was seen to be altered under the microscope.

Lactic acid produced by continuous fermentation was released from the fermented liquor by means of sulphuric acid and on removing the precipitated calcium sulphate by filtration it was evaporated to the desired concentration. This procedure showed that isolation was a separate phase of production of periodical nature. As already stated, the problem of continuous isolation as well as the control of the fermentation itself has been successfully solved according to the following principle:

The fermentation proceeded in the catode cell of a conveniently designed electro-dialysis apparatus. The lactic acid formed was continuously transported through a selectively permeable membrane into the anode cell by electric current of proper intensity and voltage. The transfer rate was controlled by a control pH-meter according to the actual acidity of the fermenting medium. In this way the free lactic acid could be maintained at the desired value of 0.5 g/100 ccm and concentrated in the anode cell to 7.5% by electric current of 40 V and 1.5 A.



STUDIES FOR CONTINUOUS FERMENTATION OF PHENOSOLVANT-EXTRACTED WASTE WATERS FROM  
BROWN COAL PYROLYSIS BY A STRAIN OF RHODOTORULA GLUTINIS

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For our experiments a waste water was used with the following main components:

|                     |      |          |
|---------------------|------|----------|
| fatty acids         | 10   | gr/litre |
| phenolic substances | 0,7  | "        |
| ammonia             | 2.4  | "        |
| neutral substances  | 2.1  | "        |
| basic substances    | 0.02 | "        |

This waste water was fortified with 1 ml 85%  $H_3PO_4$  / litre and had thereafter a pH-value between 5.2 and 5.6.

The yeast were cultivated on sugar-containing media and were used without adaptation.

For preliminary study of the problem batch fermentations were performed. It was found that a dilution to a fatty acid concentration of 2 gr/litre was most advantageous. Higher concentration experiments showed lower growth rates, altered cell forms and raising of lag time. About concentrations of 5 gr/litre growth was inhibited completely.

Artificial enlarged phenol concentrations in optimum diluted waste water showed up to 0.5 gr/litre no influence, up to 0,7 gr/litre an increased lag time but unaltered growth rate, and above 1,0 gr/litre complete inhibition of yeast growth. Under optimum conditions the maximum growth rate constant was found about  $0,18 h^{-1}$ .

Continuous experiments were accomplished in 16 litres glass vessels with a fermentation volume of 5 litres. Aeration ensued with a single hole sparger and a turbine (analogous the Waldhof system) with 800 rpm. The rate of aeration was maintained at 1.67 litres air/litre medium . min. Oxygen limitation was excluded.

The experiments were started with a batch growth above the logarithmic phase.

In these experiments the pH-value of the culture medium was alkaline - between 8.3 - 8.6. In every case after few holding times microscopic control of the culture medium showed bacteria only but no yeasts.

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For avoiding bacterial contaminations experiments were carried out at constant acid pH-value of the culture medium, purification of supplied air by cotton wool and separate addition of dilution water and original waste water. But even in these cases no yeast growth succeeded above more than three or four holding times.

Microscopic control at various times showed that the number of bacteria rose rised slowly and the number of yeasts decreased in the same manner.

It can be supposed that the growing bacteria possess a higher growth rate at lower substrate levels than the yeasts. Therefore, the steady state for the yeasts breakes down and wash-out of yeasts occurs.

The source of bacterial infection in our experiments must be the original waste water containing bacterial spores.

For technical use the cultivation of red yeasts in diluted waste waters of brown coal pyrolysis is doubtful.

APPLICATION OF CONTINUOUS-FLOW METHOD IN SOIL MICROBIOLOGY

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The continuous-flow method applied to the investigation of microbial processes in soil samples is described. The method has the principles in common with the method of heterogeneous continuous-flow cultivation of micro-organisms using a solid carrier. The method represents a special form of elective culture. It makes it possible to investigate the behaviour and the metabolic characteristic of microbial association which has accumulated in the soil as a result of continuous feeding with a certain substance. The activity of the soil microbial population can be maintained at a certain level determined by the rate of flow and by the concentration of energy source and of inorganic nutrient elements. The principle of the method and its technical arrangement are presented in detail. The applicability of the continuous-flow method to soil microbiological research is discussed and some results obtained during the investigation of microbial processes in soils by means of the method described are presented.

SITUATION AND MAIN TRENDS IN FURTHER DEVELOPMENTS OF CONTINUOUS CULTIVATION  
OF MICROORGANISMS

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A review of main results and trends in the continuous culture method and its practical application since the 1st Prague Symposium is given. A considerable increase of interest in this method may be observed; physiological states and manifestations of organisms under conditions of continuous culture were more deeply investigated, especially as far as the kinetics of some reactions are concerned. This deeper study gave rise, on one side, to a broader mathematical analysis and to a seeking for a profounder mathematical approach which would give a truer picture of the dynamics of the evolution and physiology of cultures. On the other side, it lead to the necessity of paying more attention to multi-stage processes in various fields of their applications. In spite of that indisputable progress it must be said that the method has not yet become a current tool in biological laboratories working with microorganisms; advantages of the method are practically rarely applied in the study of biosynthesis of more complicated microbial products. Also in research concerned with genetic problems and evolution of cultures the method is used only exceptionally. Some new variants of the continuous culture method were worked out, e.g. a method for soil microflora investigation, giving new possibilities of development of the method as well as of the study of biological changes in soil. The method has also found wider application in the study of animal tissue culture.

As to practical applications, the production of biomass on various substrates has been already worked out theoretically for various purposes /fodder yeast, baker's yeast, vaccines, etc./; nevertheless the study of quality as a sign of control of the physiological state of culture still lags behind the technical approach. Various bioengineering problems connected with biomass production were solved, making thus its automatic processing possible. Similarly, the continuous cultivation is being more widely used for the treatment and exploitation of industrial wastes but the physiological processes are not fully understood as yet and therefore cannot be controlled precisely and with optimal effectivity.

More attention has been also devoted to the application of the method for the production of more complicated products, such as antibiotics, acids, vitamins,

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etc. Despite of a series of studies dealing with this problem, continuous processes have not yet been introduced on industrial scale and even the possibility of economically sound application of the method in this field has been called in question. This slow development is due to an insufficient control of production processes, to an inadequate understanding of the kinetics of biosynthesis under continuous flow conditions and to the fact that little attention has been given to the theory of multi-stage processes and to further development of the necessary equipment. It would be particularly useful to consider the possibilities of combination of homogeneous and heterogeneous systems and of their applicability in such cases where growth of microorganisms and product formation are not quite parallel.

Hence the necessity follows to continue studying physiological and biosynthetic problems under conditions of continuous culture and to work out current multi-stage processes, optimal for the respective systems. This will enable us to master better the kinetics of individual processes and to ensure economical application of the method in practice, even for more complicated products.

Besides, the method should be more widely used for the study of genetics and evolution of cultures, as well as for the investigation of ecological problems, because this will be significant for the application of the method in cases when mixed population instead of sterile culture is used.

Finally, it is necessary to work out bioengineering parameters which are in better agreement with the kinetics of continuous processes and which will make possible the process automatization.

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CONTINUOUS TWO-STAGE AEROBIC CULTURE OF BAKER'S YEAST

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The morphology, chemical composition and enzyme systems of baker's yeast depend on the conditions of nutrition, aeration and other physico-chemical factors of the medium; yeast cultivation in flowing and constantly renewed medium makes it possible to stabilize the quality of the yeast cells in a desirable condition.

The continuous culture of baker's yeast in a molasses medium with an addition of nutritive salts and growth substance biotin, is carried out in two stages according to the demand of yeast cultures, which are kept in a condition of physiological activity and vegetative growth in the first stage, and in a condition of rest and stabilization of the enzyme systems in mature cells in the second stage.

In the first stage the yeast culture is maintained in a condition of continuous growth and reproduction under conditions of nutrition and aeration which are optimal for a maximum accumulation of the cell substance. Moreover, the whole complex of the enzyme systems of the cells is activated, including the zymase-maltase enzymes. With a given composition of the medium, baker's yeast can be grown with a constant increase of the cell substance. When inoculating the first vessel with especially prepared seed yeast a synchronous budding of daughter cells takes place during the first hours with increasing rate and during the 6th to 8th hour the increase of the cell substance per hour is characterized by the coefficients 1.2 - 1.25. By this time yeast cells are accumulated in the medium, differing in size and in rate of daughter-cell formation; the specific growth rate is lowered to 1.16 - 1.17. The total increase of the yeast dry weight thus amounts to 15 - 17% per hour and is maintained for a long period under conditions of complete renewal of the medium in the vessel in 6 hours.

In the second stage which is constantly fed with medium from the first vessel, stabilization of the yeast culture in the final stage of the life cycle of the young growing cells is necessary: nutrients do not enter the second vessel and the degree of aeration of the medium decreases. The yeast utilizes the rest of the nutrient substances of the medium which are difficult to assimilate and which enter the first vessel, and the yeast dry weight increases by 3 - 5%.

In the second vessel both the processes of the synthesis and dissimilation of

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the protoplasm constituents of the yeast cells are completed after 30 - 90 minutes. The nitrogen content is lowered from 9.8 to 8.2% or from 8.8 to 7.8 - 6.2% and accordingly the phosphorus content in the yeast decreases. There is also a change in the activity of the yeast enzymes: the productive activity of the yeast is lowered, the proteolytic enzymes are partly inactivated, while with suitable temperature maintained throughout the process and the correct duration of the ripening period of the yeast the zymase-maltase complex is preserved. The full renewal of the medium in the second vessel is completed in two hours.

The continuous cultivation of baker's yeast in the two-stage system ensures the production of high quality baker's yeast with good keeping properties and, high utilization of the sugars and non-sugars of the medium.



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THE BEHAVIOUR OF OIDIUM LACTIS IN CONTINUOUS CULTURE ON CARBOHYDRATES AND  
FATTY ACIDS

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As was shown by experiments which were to determine the highest possible flow rate in continuous cultivation of yeast on hexose- and pentose-containing nutrient solutions of various concentrations, there exists a relationship between the used up amount of sugar and the amount of working yeast ("yeast concentration") per time unit. A complete consumption of sugar is reached with the minimum retention time of 2.4 hours. The space-time yield of a yeast vat depends, therefore on the yeast concentration. This is correspondingly low with poor substrates. If the yeast concentration is artificially raised through yeast recycling, the retention time can be lowered to only 0.8 hours.

This "law of proportionality" found by us, reaches its upper limit only with a sugar concentration of 6% that is 3% of yeast dry weight when the population density of the yeast cells is so high that the gas-exchange and metabolism cannot take place fast enough.

In a batch "feed" experiment the demand of sugar increases correspondingly to the yeast increase without taking into account the sugar concentration up to the complete utilization of the sugar. The reaction has the character of an "autocatalysis".

Quite different is the behaviour of mycelium fungi towards carbohydrates. Continuous experiments with *Oidium lactis* gave the following results.

After obtaining a certain, very low mycelium concentration the hourly sugar demands not to increase any more with growing mycelium concentration. The consumed amount of sugar per time unit is always the same regardless of the increase of the fungi mass. This can mean that only a small part of the fungi mass takes part in the metabolism. Of decisive importance is the population density. But it is also necessary to maintain a certain minimum value of sugar, otherwise the growth is interrupted and the fungi adjust themselves for a state of starvation by the creation of *Oidia*.

The fact that through applying oxygen instead of air the stagnating growth is again brought to life, shows that lack of oxygen is a limiting factor. These results show, why *Oidium lactis* could not yet be cultivated continuously on carbohydrates on industrial scale.

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On the other hand we were able to cultivate the mycelium fungi quite continuously on a mixture of low-molecular aliphatic fatty acids. We used as nutrient the waste water of the paraffine oxidation process from the VEB Deutsches Hydrierwerk Rodleben (it contains 10 to 15 g/l of fatty acids of  $C_1 - C_8$ ) or corresponding test solutions. The fatty acids can be utilized by *Torula utilis* as well as by *Oidium lactis*. In both cases, however, is the retention time 8 to 10 hours. In pilot plant experiments the yeast was even displaced by the *Oidium* in a few days. The mycelium fungi consumed completely the nitrogen and fatty acids when the pH was about 5.0. The possibility of working with *Oidium* quite continuously using fatty acids as substrate lies probably in the fact that contrary to the uptake of sugar, in this case the resorption is brought about in the form of fatty acid anions.

CONTINUOUS YEAST PRODUCTION ON PHENOLIC SUBSTRATES

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Earlier experiments in our Institute showed that from phenolsolvan waters of browncoal coke ovens dephenolized, according to the ash-air-process, yeast can be produced without difficulties.

In order to show whether *Torula* can be adapted to low phenol concentrations in a substrate containing fatty acids we carried out experiments on models in which we produced yeast from a substrate containing acetic and propionic acid adding increasing quantities of a phenolic mixture, in this way approximating the composition of residual phenols of a phenolsolvan water using a continuous process. When adding up to 3 g/l of the phenol mixture to a substrate containing 10 g/l fatty acids, the yeast yields were constantly increasing. The phenols were also used in the synthesis of cell substance, and besides that the addition of phenol led to an increased utilization of the fatty acids. Already the addition of relatively small amounts of phenol to the substrate resulted in much larger yeast cells and the formation of chain- or tree-type cell colonies. This is not a case of mutation, as the following further multiplication on molasses led again to the formation of single cells of normal size.

It seemed to us of interest to determine in which form *Torula utilis* would grow on a carbohydrate (beet sugar molasses) - phenol-mixture. With the addition up to 5 g/l of phenol mixture to a substrate containing 10 g/l of reducing substance, the yeast yield constantly increased. The yeast itself consisted of very small cells.

In a further experiment we used yeast, which consisted of equal parts of molasses-phenol yeast (very small cells) and of fatty acid-phenol yeast (large, chain-type cells). After a six day cultivation in a fatty acid-carbohydrate-phenol-mixture the small cells became somewhat larger, their share in the total amount of yeast slightly increased. The large chain-type cells remained unchanged.

When we transferred a yeast from a molasses-medium to a fatty-acid-carbohydrate-phenol-mixture, after a short test period we could notice a change in the cells which at the beginning of the experiment were uniform and now were differentiated into large, chain-type cell colonies and small individual cells. Such a yeast mixture when returned to the normal molasses substrate, changed back in its appearance to the typical molasses yeast.

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It proved possible to produce yeast from phenolsolvan water from the VEB Grosskokerei Lauchhammer under continuous culture conditions without difficulties. Another 600 mg/l of phenol mixture could then be added to the original substrate which contained 410 mg/l of residual phenols. Also on this substrate large cells of yeast were formed.

One litre of phenolsolvan water, containing 9 - 10 g/l of fatty acids yielded about 35 g of dry yeast. In this reaction the fatty acids and phenols were almost completely used up. Of the 2.04 g N/l however 1.7 g N/l remained in the yeast free filtrate. But such a high percentage of nitrogen is in many respects harmful for the receiving stream. We therefore tried to carry out a deammonization in a microbial way, as the residual nitrogen in the yeast free filtrate can be a N-source for the yeast forming in further organic compounds. All technical waste products from which yeast can be produced can serve as carbon-source; the C-source must be in a concentrated form, as the phenolsolvan water does not serve only as N-source, but also as a means of dilution. For our experiments we used molasses of beet sugar. In a first test group, 2 per cent of RS (= 4% molasses) were added to the phenosolvan water of common composition. Using a retention time of 6 hours, fatty acids, red. substance and phenols were consumed. From 1 litre of substrate about 14 gr of dry yeast could be isolated, in the filtrate without yeast remained about 1 g N/l. To this filtrate 2.4% RS (= 4.8% molasses) were added in a second test group. Using a retention time of 6 hours the reducing substance was used up completely, in the filtrate without yeast remained then still about 0.4 g N/l. From 1 litre of substrate about 13 g of dry yeast could be isolated.

CONTINUOUS PRODUCTION OF YEAST AS A MEANS OF UTILIZING "UNPHYSIOLOGICAL"  
SUBSTANCES

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Up to 1936 it was generally agreed that pentoses could not be utilized by yeast. In 1936 Seidel, a co-worker of H. Scholler, succeeded to make yeast from wood-sugar, which consisted mainly of d-xyloses.

In the same year yeast was made from sulphite liquors of beech-wood in the former I.G. Werken, Ludwigshafen und Wolfen. Results of feed experiments published in 1938 by Lechner who used the Fink standard apparatus showed that when using *Torula* with glucose-xylose-mixtures a decrease of xyloses could be noted but no corresponding increase of yeast. With the continuous process, however, we were able to make yeast of sulphite liquors of beech-wood up to 6% RS, while Lechner later discovered that in the feed experiment with xyloses the optimum concentration 1.5%, using sulphite liquors of beech-wood amounts to 1.2%. The difference in these results, especially concerning the very short retention time, is probably caused by the fact that Scholler and we used for the first time the continuous method, while the other authors used batch methods. The pentose yeast-forming can be considered as the first technical use of an "unphysiological substrate" for the microbial protein synthesis.

The technical introduction of the continuous yeast production process from sulphite alcohol stillage by us and H. Butschek in 1940 is based on the utilization of pentoses and non-sugar substances.

As a logical continuation of these studies we tried to find out by using the continuous process, to what extent "unphysiological substances" known even as "cell poisons" can be used for the microbial synthesis. These studies are important from the point of view of treatment and utilization of industrial waste waters.

In the continuous flow of the unphysiological substrate or the "cell poison" its concentration in the culture is always only very small, when it is utilized at all.

Whereas acetic acid is still utilized by the *Torula* which has been adapted to the substrate in the feed experiment, difficulties are encountered with the longer chain fatty acids, which are greater, the greater the number of carbon

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atoms. Beyond  $C_6$  acid (capronic acid) the toxicity of the fatty acid rises by jumps. Formic acid is also a cell poison and normally inhibits any growth of fungi.

In the continuous process acetic acid can be very quickly changed into cell substance as soon as after 3.4 hours, the same applies to a mixture of 10 g of acetic acid and 5 g of formic acid per litre in a period of 7 hours. In this case the formic acid is not utilized in the cell substance synthesis, and as a result of its "detoxication" by the yeast a longer period is necessary. The economic coefficient is also slightly less favourable than in the case with acetic acid and fatty acids  $C_3$  to  $C_6$  alone.  $SO_2$  seems to have a similar effect. During the paraffin oxidation in the VEB Deutsches Hydrierwerk Rodleben there is a great amount of waste water, containing 20 - 30% of the fatty acids as formic acids, the same amount of acetic acid and the rest in increasingly smaller parts  $C_3$  to  $C_9$  of fatty acids. These fatty acids - except for the formic acid - are completely utilized by *Torula* in model experiments as well as in original waste waters with an economic coefficient of 40, discounting the formic acid, with a retention time of 8 - 10 hours. Phenols as carbon source are also utilized in a mixture of fatty acids or sugars with phenols /see paper of M. Lorenz/.

Of great practical importance is the finding that when producing yeast from beet sugar molasses with a great prolongation of the necessary retention time through the re-use of the filtrate 8 or more times to dilute the concentrated molasses, i.e. through the recycling of the filtrate, the non-sugar substances of the molasses e.g. amino acids, can be utilized, which normally cannot be assimilated by yeast. An increase of 40% of yeast is thus obtained.

PRELIMINARY STUDIES FOR CONTINUOUS PRODUCTION OF DEXTRAN

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Microbial syntheses are more complex processes than cell growth. Therefore, application of continuous methods will be successful only, if the course of the main particular processes is known.

Dextran synthesis is an example for such a complex process. Early it was found by Hehre that dextran-producing organisms release an enzyme synthesizing dextran independently from organisms. Therefore, the dextran synthesis is subdivided into three particular processes:

- growth of organisms
- production of enzyme by organisms
- synthesis of dextran by enzyme

For determination of type and rate of the first process the method of Jones was used. It is based on the balance equation of microorganisms growth and flow under steady state conditions. The same method is well applicable for determination of type and rate of the second process using the balance equation for product synthesis and flow. Both processes showed the same type of reaction: positive autocatalytic and positive pseudoautocatalytic respectively.

These processes can be performed both in one continuous step. Thereby technical system has to be the ideal mixed vessel.

A difficulty for continuous production of enzyme arises by the fact that release of enzyme only occurs in the presence of notable concentrations of sucrose. The concentration of sucrose added to the continuous system must be chosen in a manner warranting release of enzyme as well as a minimum amount of sucrose in the medium for in the first step notable dextran synthesis was not wanted.

The dependence of enzyme stability from environmental conditions such as pH and temperature was taken into account.

Under optimum conditions of microbial growth these influences are negligible.

The mechanism of producing dextran by the enzyme was described by various authors. Up to a concentration of 5% of sucrose the enzyme reaction is of first order; higher concentrations showed zero order.

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For continuous production it is advantageous to work within concentrations warranting a zero order reaction. In this case it is quite the same, if an ideal mixed vessel or a plugflow system is used. Practical considerations led to application of the mixing system.

The last process has been studied for the present without consideration of controlled molecular-weight synthesis. Such studies are an object of further work.

It is shown that only application of methods well known in chemical reaction engineering leads to successful continuous microbial syntheses.

STUDIES ON BIOSYNTHESIS OF 6-AZURACIL RIBOSIDE BY ESCHERICHIA COLI IN  
A CONTINUOUS MULTISTAGE PROCESS

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Studies of 6-azauracil riboside (AzUR) biosynthesis by *Escherichia coli* have shown that in synthetic medium batch culture the major part of the process occurs in the initial logarithmic phase. Attempts have been made in our laboratory to establish favourable conditions for the AzUR biosynthesis in continuous culture assuming that fresh feeding of proper composition and adequate flow rate helps to preserve the producing capacity of the organisms for a long period of time. A prolonged AzUR biosynthesis, however, cannot be carried out in a single-stage homocontinuous fermentation for several reasons. In order to attain a state of culture such as to correspond to the beginning of the logarithmic phase the dilution rate is required to be close to  $D_{max}$ . The addition of azauracil (AzU) inhibiting the growth of the cells results in "wash-out" of microorganisms. When the flow rate has been diminished, then in the presence of AzU a new "resistant" culture losing its ability of producing AzUR comes into existence.

Favourable conditions for the AzUR biosynthesis in continuous culture can be provided only by the multistage process. In the first stage the inoculum is prepared and then fed together with the fresh medium into the next stage. Now the dilution rate close to  $D_{max}$  may be used without any risk. At this stage the properties of the culture are comparable with those at the beginning of the initial logarithmic phase. Even the presence of AzU, which is added under the conditions mentioned, no wash-out of the microbial population is observed. Nevertheless, the inoculum grown continuously in a synthetic medium gradually loses its ability to produce AzUR. The activity of the impaired cells is fully restored by two passages on blood agar or in broth. Therefore it is necessary to prepare the inoculum continuously in a rich nutrient solution, e.g. in broth. Its composition must be such that on dilution with the synthetic medium in the second stage the concentration of the pyrimidine type components should drop to a value which could not substantially interfere with the ribosidation of the AzU.

The yield of AzUR is dependent on the physiological properties of the inoculum. The inoculum prepared at the dilution rate of about 0.138 gave the highest AzUR production. This value applies both for the inoculum grown in broth and in synthetic media.

Finally, it has to be pointed out that the AzUR concentration owing to the great dilution rate at the stage receiving the addition of AzU does not reach that of the batch cultures. If higher accumulation of the product in the medium is desirable it is necessary to put in an additional stage.

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### CONTINUOUS FERMENTATION OF CHLORTETRACYCLINE

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In a one-stage or two-stage pilot-plant fermentation unit, continuous fermentation of chlortetracycline was performed using the strain Streptomyces aureofaciens 84/54 in a medium containing sucrose (4%), soy-bean-meal (3%), corn-steep liquor (0,25% - calculated on dry matter basis), ammonium sulfate (0,5%), sodium chloride (0,2%), monobasic potassium phosphate (0,01%), molasses (0,2%) and benzyl thiocyanate (4 mcg./ml.). Fermentors with a working capacity of 10 - 15 litres were equipped with turbine-type agitators, spargers and 4 baffles. The volume of medium in both fermentors was the same. The agitating velocity was 400 rpm., the air flow 0,5 vol. per volume of culture per min. The oxygen transfer rate, determined by the method of Cooper, Fernstrom and Miller (1944), was 1.5 ml. O<sub>2</sub> per ml. per hour. During the fermentation, determinations were made regularly of the concentration of chlortetracycline, sucrose, NH<sub>3</sub>-nitrogen, aminonitrogen and inorganic phosphorus.

The following problems were studied:

1. The influence of the dilution rate in the first stage on the concentration of chlortetracycline and of the individual nutrients.
2. The influence of the dilution rate in the second stage on the concentration of chlortetracycline and of the individual nutrients.
3. Chlortetracycline production in samples withdrawn from the first and second stage during continuous cultivation and transferred to shaking flasks.

The flow of fresh medium was started 24 hrs. after inoculation. When the addition was begun later, the steady state was reached only after a longer period of cultivation.

The relationship between the concentration of the antibiotic and of the individual nutrients and between the dilution rate in the first and second stage is tabulated below:

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| Dilution rate | Cultivation stage | CTC mcg./ml. | Sucrose mg./ml. | NH <sub>3</sub> -N mg./ml. | NH <sub>2</sub> -N mg./ml. | Phosphorus mcg./ml. | pH   |
|---------------|-------------------|--------------|-----------------|----------------------------|----------------------------|---------------------|------|
| 0,049         | 1                 | 872          | 23,41           | 0,51                       | 0,219                      | 0 <sup>x/</sup>     | 6,6  |
| 0,049         | 2                 | 1936         | 14,80           | 0,29                       | 0,209                      | 0 <sup>x/</sup>     | 6,0  |
| 0,092         | 1                 | 656          | 34,57           | 0,79                       | 0,250                      | 0                   | 6,39 |
| 0,092         | 2                 | 1018         | 28,20           | 0,64                       | 0,223                      | 0                   | 6,29 |
| 0,138         | 1                 | 357          | 35,09           | 0,87                       | 0,251                      | 2,0                 | 6,81 |
| 0,138         | 2                 | 712          | 29,52           | 0,70                       | 0,219                      | 0                   | 6,72 |
| 0,153         | 1                 | 263          | 37,03           | 0,92                       | 0,224                      | 4,4                 | 6,65 |
| 0,153         | 2                 | 503          | 31,49           | 0,79                       | 0,214                      | 0                   | 6,57 |

x/ Concentration of phosphorus in feed - 20 mcg./ml.

Values in the table are mean values in the steady state.

At dilution rate 0,049 hr<sup>-1</sup> and with the concentration of inorganic phosphorus in the inflowing medium higher than 30 mcg./ml., the concentration of the antibiotic gradually decreased during cultivation - in the first stage it dropped from 1250 mcg./ml. after 50 hrs. to 100 mcg./ml. after 95 hrs.; in the second stage it dropped from 2500 mcg./ml. after 50 hrs. to 400 mcg./ml. after 140 hrs. When other dilution rates were used, the concentration of the antibiotic was held constant. But even at the dilution rate 0,049 hr<sup>-1</sup> it proved to be possible to attain the steady state of the antibiotic concentration by limiting the inorganic phosphorus concentration in the feed to 20 mcg./ml.

Chlortetracycline production, either in samples withdrawn during continuous cultivation from both stages and inoculated into fresh medium, or in samples in which fermentation was allowed to proceed in flasks, was the same at all dilution rates (4000 - 4500 mcg./ml.).

It is evident from the table that the most advantageous process is two-stage fermentation operated at the dilution rate 0,049 hr<sup>-1</sup> in both stages, yielding on the average 2000 mcg./ml. of chlortetracycline in the second stage. Batch cultivation of the same strain in the same medium yielded an average of 4500 mcg./ml. in 70-hr. runs. The rate of antibiotic production per hour amounts to 50 mcg./ml./hr. in batch cultivation, 100 mcg./ml./hr. in continuous cultivation. The continuous method is therefore about twice as productive as the batch method. So far, its drawback lies in incomplete utilization of nutrients which could be improved, however, by using a more diluted medium or a lower dilution rate.

THE SYNTHESIS OF PIGMENTS IN CONTINUOUS CULTIVATION OF STREPTOMYCES AUREOFACIENS

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In a previous communication (Sikyta B., Slezák J., and Herold M., Appl. Microbiol., 9 : 233, 1961) we have described our observation that during continuous cultivation of the strain Streptomyces aureofaciens in a synthetic medium with sucrose as limiting factor, striking changes in chlortetracycline production and pigment formation occur when the dilution rate is changed. At dilution rate values higher than  $0,05 \text{ hr}^{-1}$  the antibiotic and the pigments are both produced, but neither of them is synthesized at dilution rates lower than  $0,05 \text{ hr}^{-1}$ . This phenomenon was shown to be reversible.

In our present work we are dealing with a more detailed study of this phenomenon and its causes. Chlortetracycline concentration was assayed by the biological method, pigment production by streptophotometric determination of absorbance in the wavelength region 200 - 500 mμ, at alkaline, neutral and acidic pH values (the pigments change color with changes in pH). Quantitative determination of antibiotic and pigment concentrations confirmed previous observations, where pigment production was only estimated visually.

The rate of production of chlortetracycline and of pigments was studied both during batch growth and during continuous growth. Continuous cultivation experiments were performed in such a way that, when batch growth was finished, the culture was diluted at a rate lower than  $0,05 \text{ hr}^{-1}$ , which resulted in a gradual decrease in the synthesis of the antibiotic and pigments; mycelium lost pigmentation and pigments were released into the medium. After some time the dilution rate was again raised above  $0,05 \text{ hr}^{-1}$ , whereupon the synthesis of the antibiotic and pigments was resumed. Such changes in dilution rate were repeated several times.

The experiments brought several new observations:

1. The production of the antibiotic and of pigments does not start again immediately after the increase in dilution rate, but only after a certain lag-period.
2. Changes in absorbancy in the wavelength region given above in samples withdrawn at regular time-intervals, both in batch and in continuous cultivation, lead to the conclusion that the individual single pigments

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are synthesized independently and separately.

3. The time necessary to attain the steady state of the culture depends on whether dry weight of mycelium, antibiotics titer or absorbancy of pigments is taken as criterion for characterization of the steady state. The steady state of constant dry weight of mycelium was reached first, constant antibiotic concentration was reached only after a considerably longer period. A steady state characterized by constant absorbancy was not reached until a very long time.

The following possible explanations for changes in the synthesis of the antibiotics and pigments were considered the most probable:

- growth of the organism at a very low concentration of sucrose,
- production of inhibitors of the pigment synthesis which might be accumulated at low dilution rates,
- lack of trace elements and growth factors at a low dilution rate, and
- genetic changes in the population.

By means of a series of cultivation experiments all of these possibilities have been eliminated, with the exception of genetic changes. The results obtained so far lead us to the conclusion that the phenomenon is caused by selection of at least two strains in a heterogeneous population. A nonpigmented part of the population, producing no antibiotic, has a higher growth rate at low concentrations of limiting substrate (at low dilution rates), whereas a pigmenting and antibiotic-producing part of population has a higher growth rate at a higher concentration of the limiting substrate-sucrose (at a higher dilution rate).



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CONTINUOUS FERMENTATION OF NON-STERILE STARCH CONTAINING MEDIA

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A continuous flow process suitable for fermentation of ethyl alcohol from starch containing materials and for acetone-butanol production has been worked out, experimentally tested and used on commercial scale.

From the laboratory, pilot plant and plant scale experiments the following conclusions were drawn:

- (a) In a battery consisting of several fermenters the medium flows irregularly: It often occurs that the flow rate of the fresh medium is too high which results in losses due to incomplete sugar utilization. The fermented medium on the other hand may be retained in the fermenters for a prolonged period of time and turn sour, thus causing contamination of the medium.
- (b) Contaminating microflora (mostly lactic acid bacteria) was found to develop first in the head fermenter and then to spread into the following fermentation vessels of the battery. Increase in acidity does not appear first in the head fermenter but in the rear vessels because in the head fermenter the medium is constantly diluted by inflow of fresh medium with lower acidity and the retention of fermented medium here is smaller than in the following vessels. Alcohols accumulating in the course of the fermentation process do not inhibit the growth of lactic acid bacteria, which brings about a gradual increase of the fermented medium acidity in the rear fermenters of the battery. Preventive sterilization of the fermentation equipment carried out every 72 hours prevents the growth of acid forming bacteria in the medium and secures aseptic conditions of the fermentation process.
- (c) Continuous fermentation of carbohydrates by yeast and acetone and butyl alcohol bacteria in interchangeable media was investigated. It was proved that both fermentation processes are governed by the mononuclear reaction laws. Reaction rate constants under various fermentation conditions were calculated.
- (d) In order to attain optimal population density ( $1.10^7$  yeast cells or  $7.10^9$  bacteria per 1 ml of the medium) at the very beginning of the

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fermentation, the medium must be inoculated with a large quantity of seed yeast or acetone and butyl alcohol bacteria, the portion of mature cells in the inoculum being not less than 80%.

- (e) The inflow rate of the medium into the head fermenter must correspond to the rate of carbohydrate utilization in the fermentation battery, while the properties of yeast or bacteria in the head fermenter must be such as to correspond to the stationary growth phase. Thus, the transition to the lag phase and to the early phase of logarithmic growth occurring in batch and semicontinuous fermentations with small quantity of seed culture and high feed rate, is precluded.
- (f) In ethyl alcohol fermentation by yeast the exchange of medium in the head fermenters is 1.3 volume per day and in the acetone-butyl alcohol fermentation 2.5 volumes per day.

Introduction of continuous fermentation method in alcohol production made it possible to increase the productivity by 15% as compared with the batch process. The technological properties of fermented medium improved and the degree of carbohydrate utilization increased by 45%.

In acetone and butyl alcohol industry the increase in productivity was 20%, technological qualities of the fermented medium improved and the degree of ideal starch utilization increased by 0.3% as compared with the semicontinuous battery method.